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Award Number DAMD17-98-1-8283

TITLE: Expression and Function of FGF-8 During Murine Mammary Gland Development and Tumorigenesis

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REPORT DATE: May 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*

2. REPORT DATE
May 1999

3. REPORT TYPE AND DATES COVERED
Annual (13 Apr 98 - 12 Apr 99)

4. TITLE AND SUBTITLE
Expression and Function of FGF-8 During Murine Mammary Gland Development and Tumorigenesis

5. FUNDING NUMBERS
DAMD17-98-1-8283

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Fibroblast growth factors (FGFs) are potent growth factors that have been shown to play crucial roles in developmental processes and have been correlated with tumorigenesis. FGFs make up a family of eighteen genes (FGF 1-18) coding for secreted ligands and four genes coding for the FGF receptor. Several recent studies have suggested a correlation between FGF8 expression and tumorigenesis. FGF8 can transform NIH 3T3 cells, is overexpressed in human prostate and breast tumors and is expressed in several tumor cell lines. FGF8 is the only FGF family member that has been shown to be overexpressed in human breast cancer. Experiments in our laboratory suggest that FGF8 is expressed at very low levels during virgin development and increases during pregnancy. Overexpression of FGF8 was detected in several mammary gland tumors derived from hyperplastic alveolar nodules and the DIM3 mammary tumor cell line. This is in contrast to other FGF family members that are generally down regulated during pregnancy and in tumors. The objective of this proposal is to understand the mechanism of FGF8 action in mammary tumorigenesis and development using mammary reconstitution experiments. Towards this end a system of inducible FGFR activation has been developed to elucidate the role of receptor signaling in mammary tumorigenesis.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18 298-102

USAPPC V1 00

FOREWORD

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Introduction

Fibroblast growth factors (FGF) have been described as being potent growth factors involved in many developmental events during embryogenesis. FGFs play a role primary mesoderm induction, limb bud outgrowth and patterning, and lung alveogenesis. During these developmental events the expression of FGFs are restricted and regulated. The aberrant expression of FGFs outside the confines of developmental events has been correlated with tumorigenesis. The oncogenic roles of FGFs in mammary tumorigenesis were initially identified by the finding that mouse mammary tumor virus (MMTV) insertion can cooperatively activate Wnt-1 and FGF3 in tumors of MMTV infected mice. Two other FGF family members have also been identified that cooperate with Wnt-1 to induce mammary gland tumorigenesis: FGF4 and FGF8. We are interested in elucidating the function of aberrant expression of FGFs in mammary tumorigenesis. Overexpression of several FGFs has been shown to induce mammary tumorigenesis in mouse models and has been postulated to play a role in human breast cancer. Understanding the mechanism of FGFs and FGF receptors (FGFR) in breast cancer may provide insight for new therapeutics derived to block FGF/receptor interactions or receptor tyrosine kinase activity. The goal of this project is to understand the mechanism of mammary oncogenic FGF8 and FGF receptors in tumorigenesis.

Body

We have modified our initial proposed statement of work to address new developments in the field. These developments include a paper that describes the oncogenic ability of FGF8 when overexpressed in the mammary gland of transgenic mice (Oncogene 1998 17, pg 2711-1717). Eighty-five percent of the transgenic mice developed adenocarcinomas within a year. Several of the transgenic mice also developed metastasis to the lung. Since this study demonstrated the oncogenic potential of FGF8 in mammary tumorigenesis our proposal has been modified to avoid redundancy on the descriptive characterization of FGF8s oncogenic ability. In addition, due to anticipated problems with the original objective #4 this project has not been pursued (see below). In light of these results the focus of our project has been shifted to focus more on the elucidation of the signaling pathways of FGF8 and FGFRs that may be involved in their oncogenic role during mammary gland tumorigenesis. The experiments are designed to examine FGF oncogenesis more mechanistically rather than descriptively. To this end we have developed a system of inducible FGF activation that will be used initially to study the signal transduction involved in transformation of mammary epithelial cell lines. This system will also be used in transgenic mice to develop an *in vivo* model for FGF induced transformation of the mammary gland. The objective is to understand the mechanism of FGF8 and FGFRs in mammary gland tumorigenesis. The system of inducible FGFR activation will allow us to understand two important aspects of FGF signaling in mammary gland tumorigenesis. First, it will address the issue of the signaling effects of FGFR homo and heterodimerization. Second, signaling comparisons can be made from different FGFR dimers with that of FGF8. Since FGF8 may interact with members of all four FGFR gene it will be important to not only understand FGF8s oncogenic potential but also through which receptor FGF8 is mediating its effects. The proposal modifications mainly effect technical objectives two, three and four and are described in further detail below.

The modified technical objectives are as follows:

1) To determine the spatial and temporal expression of Fgf-8 during normal mammary gland development and in tumors.
2) To examine the expression and function of genes that may be regulated by Fgf-8 and FGFRs in the normal mammary gland and tumors.
3) To characterize the cooperative potential of Fgf-8 and beta-catenin in mammary tumorigenesis.
4) Determine the oncogenic potential of the FGFRs in mammary gland tumorigenesis.

Technical Objective #1: FGFR and FGF8 protein and RNA analysis
Technical difficulties have occurred with the use of FGF8 antibodies that we have developed. The antibodies have proven to be cross-reactive with several proteins isolated from tissue extracts making the results uninterpretable and the antibodies are unlikely to be useful in further experiments. We have also purchased or obtained FGF8 antibodies from several sources for this project. These antibodies have also been unsuccessful at detecting FGF8 protein isolated from tissue samples in Western blot analyses. Several biochemical techniques including heparin Sepharose purification of protein extracts, modification of the extraction process, immunoprecipitation , and affinity purification of eight antibodies made against different epitopes have failed to produce quality Western blot or immunohistochemistry results. Until new high titer and highly specific antibodies are developed or can be acquired by our lab we will not be able to characterize FGF8 protein expression as stated in technical objective one. To avoid this problem in our functional studies we have made FGF8 constructs that contain the myc and flag epitope tags.

FGF8 and FGFR RNA expression has been examined by RT-PCR analysis from extracts from developing mammary tissue and tumors. These results were initially described as preliminary data in the proposal and progress in the past year has been made at developing probes for RNase protection assays (RPA) and optimizing the conditions. Advantages of RPA analysis include quantitative measurement of FGF8 RNA and the use internal controls. The data from the RPA analysis correlates well with the initial RT-PCR experiments showing FGF8 overexpression in tumors derived from hyperplastic alveolar nodules. Although RPA analysis is sensitive enough to detect FGF8 for analysis of tumor RNA samples it is not sensitive enough for detecting FGF8 in the developmental samples. For analysis of developmental regulation of FGF8 RNA in the mammary gland RT-PCR and poly-A northern blot analysis will be used. Tissue isolated from different developmental stages in the mammary gland including virgin, pregnant and lactating has been collected for these experiments.

The level of FGF10 and Shh RNA have also been examined by Northern blot analysis. We have not detected a change in their RNA levels for these factors in the FGF8 overexpressing tumor samples. FGFR RNA levels have been studied by RT-PCR. All four FGFR gene family members have been detected in the mammary gland.

Technical objective #2: FGF8 and FGFR regulated genes.

To understand the mechanism of FGF8 and FGFR action in mammary tumorigenesis we have developed a system of FGF independent but drug inducible activation of the FGFR. This method utilizes the binding interactions of FK506 (a lipid soluble immunosuppressant drug) with the FK506 binding protein (FKBP). The FKBP domain that interacts with FK506 has been cloned into the FGFR intracellular domain. The FGFR extracellular ligand binding domain has been removed preventing interactions with FGFs. These modified FGFR constructs also contain an amino terminal myristlation sequence and a carboxyl terminal HA epitope tag sequence. To induce dimerization and activation of these constructs the drug FK1012 is used. FK1012 is a molecule composed of two FK506 domains linked together. The two linked FK506 molecules are capable of interacting with two FKBP domains on two different modified FGFRs. This causes two modified FGFRs to become proximally localized, trans-phosphorylated and activated in a drug inducible manner. This system has been initially characterized in NIH 3T3 cells. Our results show that the modified FGFR constructs, FVR1 (FGFR1), FVR2 (FGFR2), FVR3 (FGFR3), are capable of dimerizing, transphosphorylating, activating the MAPK pathway, inducing an AP1 reporter and activating AKT in a FK1012 dependent manner. These results are expected in a normal response to FGF ligand. In the past year all four genes in FGFR family have been cloned into the modified constructs in at least two orientations. These constructs have been well characterized in NIH 3T3 cells and are now being used to study FGF signaling in mammary epithelial cell lines.

Our current progress towards this aim has been to develop and characterize the constructs. We are currently studying and comparing the signaling pathway for FGF8 and FGFR homodimers. Our most interesting data has been an observation that both FGF8 and some FGFR homodimers can provide survival signals to cells under conditions of serum starvation. We are now investigating the anti-apoptotic effects of FGF8 treated cells and cells expressing the FV receptor variants. Data from these experiments will also be used in further characterization of the aims in technical objectives three and four.

Technical Objective #3: FGF8 and beta-catenin cooperation in tumorigenesis

Our initial proposal for this objective was to study FGF8 transformation of the HC11 mammary epithelial cell line using an *in vitro/in vivo* model. We have made stable cell lines that express Wnts and FGFs and transplanted these cells and control nontransfected cells back into the cleared fat pads of syngeneic host mice. These experiments have provided variable results due to the genetic instability of the HC11 cells. Most transplants contained some degree of differentiated alveolar buds localized at the site of transplantation. There was, however, a high incidence of hyperplasia and abnormal

growth surrounding the transplants including in the control experiments. Our conclusion from over thirty transplants is that the technique is highly variable and not useful for functional studies of FGF8.

We have been developing a new mammary reconstitution system to use for our functional studies of FGF8. This method uses retrovirus infection of mammary primary cultures that are then transplanted back into the cleared fat fad of syngeneic mice. Mammary reconstitution from primary cultures has several advantages over the HC11 cell system. The method has been well described in the literature and can produce functional outgrowths that fill most of the fat pad. This system has been used to successfully describe the oncogenic potential of HGF, WNT1, and RAS in the mammary gland. In the past year we have constructed retroviruses that overexpress FGF8, stabilized beta-catenin, green fluorescent protein, and beta-galactosidase. We have optimized the method in our lab for primary culture production and efficient retrovirus infection. At this point we have transplanted 22 animals with mammary primary culture cells that express FGF8 alone, beta-catenin alone, FGF8 and beta-catenin together and beta-galactosidase alone. The results from this initial experiment are expected in May 1999..

This objective has been modified from the proposed objective to address the question of how FGF8 can cooperate with other growth factors in mammary tumorigenesis. Several studies have shown that FGF8 can cooperate with WNT1 in mammary tumorigenesis. The goal of this project is to elucidate FGF8's cooperative ability with the WNT signaling factor beta-catenin. An attractive model for cooperative oncogenesis is that at least two events are necessary for tumorigenesis: survival signals and proliferative signals. Applying this model to the cooperative oncogenesis observed with WNTs and FGFs we hypothesize that FGFs provide survival signals and WNTs proliferative signals. This hypothesis will be tested by studying changes in proliferation and apoptosis in the transplanted outgrowths.

Technical Objective #4: Developing transgenic mice that express inducible FGFRs in the mammary gland.

The aim of this objective is to determine which FGFRs can function in mammary gland tumorigenesis *in vivo*. The constructs for inducible FGFR activation described in technical objective #2 will be used to develop the transgenes. Progress on this project has

been to construct the transgenes that express FVR1 and FVR2 under the MMTV LTR. The transgenes consist of the MMTV LTR promoter followed by the KCR element (beta-globin intron) and the FVR1 and FVR2 constructs. This MMTV LTR driven construct should produce low levels of the transgene during ductal morphogenesis and high levels during pregnancy. FGFR activation can be induced in the transgenic mice during different periods of development by providing FK1012 to the mice in their drinking water. Any abnormalities in development including hyperplasias and tumors will be studied for proliferation and apoptosis levels and compared with our results from technical objective #2.

Changes to the proposed Technical Objective #4

This original proposed objective was to make a targeted knock-out of FGF8 in the mammary gland. The proposal was to develop mice that express the Cre recombinase in the mammary gland under the beta-casein promoter. These mice would then be bred with the floxed FGF8 mice developed by in Dr. Gail Martin's lab. The offspring from these mice would then be bred to initiate pregnancy inducing Cre expression in the mammary gland and causing recombination at the FGF8 lox sites. After further discussions with our collaborator (Dr. Gail Martin, UCSF) on this project we have determined that due to several potential difficulties the probability for success would be low. Since FGF8 functions in a paracrine manner a very high recombination rate would be required. This is important to prevent the paracrine action of FGF8 secreted from wildtype cells from compensating for the FGF8 loss in knock-out cells. Unfortunately, most of the existing transgenic lines expressing Cre recombinase in the mammary gland display patchy expression patterns. Furthermore, other FGF family members expressed in the stroma and mammary epithelium may compensate for the loss of FGF8 during development.

Key Research Accomplishments

-Technical objective #1: Confirmed initial RT-PCR data describing the overexpression of FGF8 in mammary tumors using RNase protection assays.

-Technical Objective #2: Developed and characterized an inducible system for FGFR activation.

-Technical Objective #3: Began mammary gland reconstitution experiments using a retroviral method for overexpression of FGF8 in transplanted mammary primary cultures.

-Technical Objective #4: Constructed transgenes for inducible activation of FGFR1 and FGFR2.

Reportable Outcomes

Data from this project has been presented at two institutional symposiums.

Graduate Student Symposium, March 12, 1999. Houston, Texas. Poster Presentation.

Cell and Molecular Biology Symposium, January 22, 1999. Galveston, Texas. Speaker

Statement of Work
(updated 5/23/99)
Fgf-8 Expression and Function during Murine Mammary Gland Development and Tumorigenesis

Task 1 : To determine the temporal and spatial expression of Fgf-8 and FGFRs during mammary gland development and in tumors. (months 1-12)

a. Finish preliminary experiments on Fgf-8 expression during mammary gland development and in tumors. (months 1-8)

b. Examine the expression of FGFRs during normal mammary gland development. (months 1-8)

c. Characterize expression of FGFRs in mammary gland tumors.(months 1-10)

Task 2 : Examine the expression and function of genes that may be regulated by Fgf-8 and FGFRs in the normal mammary gland and tumors (months 4-15)

a. Determine the temporal and spatial expression of Shh and Fgf-10 during mammary gland development. (months 4-15)

b. Examine the expression of Shh and Fgf-10 in mammary gland tumors. (months 4-15)

c. Develop system of inducible activation of the FGFR. (months 8-14)

d. Identify and characterize genes that are regulated by FGFR activation in mammary epithelial cells. (months 14-30)

Task 3 : Characterize Fgf-8 tumorigenicity in the mammary gland. (months 1-24)

a. Clone Fgf-8 isoforms into retrovirus vectors. (months 5-10)

b. Optimize primary culture conditions and retrovirus infection efficiency. (months 8-13).

c. Transplant Fgf-8 infected primary cultures into syngeneic mice. (months 12-24)

d. Prepare and characterize outgrowths. (months 16-24)

Task 4: Determine the oncogenic potential of the FGFRs in mammary gland tumorigenesis.

a. Construct FVR1 and FVR2 transgenes. (months 12-14)

b. Inject transgenes. (months 14-15)

c. Characterize transgenic mice. (months 16-36)